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SURVEY AND SUMMARY

DNA damage, cellular senescence and organismal ageing: causal or correlative?

Jian-Hua Chen*, C. Nicholes Hales† and Susan E. Ozanne

Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Cambridge CB2 2QR, UK

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ABSTRACT

Cellular senescence has long been used as a cellular model for understanding mechanisms underlying the ageing process. Compelling evidence obtained in recent years demonstrate that DNA damage is a common mediator for both replicative senescence, which is triggered by telomere shortening, and premature cellular senescence induced by various stressors such as oncogenic stress and oxidative stress. Extensive observations suggest that DNA damage accumulates with age and that this may be due to an increase in production of reactive oxygen species (ROS) and a decline in DNA repair capacity with age. Mutation or disrupted expression of genes that increase DNA damage often result in premature ageing. In contrast, interventions that enhance resistance to oxidative stress and attenuate DNA damage contribute towards longevity. This evidence suggests that genomic instability plays a causative role in the ageing process. However, conflicting findings exist which indicate that ROS production and oxidative damage levels of macromolecules including DNA do not always correlate with lifespan in model animals. Here we review the recent advances in addressing the role of DNA damage in cellular senescence and organismal ageing.

INTRODUCTION

It has long been suspected that ageing is closely linked with damage. Indeed cellular macromolecules are constantly exposed to both extrinsic and intrinsic damage. Sources of extrinsic damage include UV irradiation and other environmental toxic agents whereas intrinsic insults principally consist of reactive oxygen species (ROS) and spontaneous hydrolysis (1,2). ROS are produced during normal cellular metabolism, particularly by respiration in mitochondria, and when ROS production exceeds the capacity of detoxification, can cause oxidative damage to macromolecules including DNA. There is an emerging consensus that a progressive and irreversible accumulation of oxidative damage contributes to impaired physiological function, increased incidence of disease and thus impacts on the ageing process (3,4).

Although ageing may involve damage to various macromolecules, for those that can be replaced by their fast turnover, damage may not accumulate and therefore may not be critical. DNA, on the other hand, is the prime information molecule of the cell and nuclear DNA in particular must last the lifetime of the cell. Therefore, DNA damage represents a critical threat to cell function. If DNA damage is severe or its accumulation exceeds its elimination by DNA repair mechanisms, cellular senescence or apoptosis will occur and this may contribute to the ageing process.

Experimental approaches that aim to understand the importance of DNA damage in the ageing process include (i) observations of cumulative occurrence of DNA damage in ageing cells *in vitro* as well as in tissues of aged organisms including humans, (ii) non-genetic interventions that influence oxidative stress, DNA maintenance and lifespan and (iii) genetic manipulations that either enhance resistance to oxidative stress or compromise DNA integrity and the consequential effects on lifespan. Here we review *in vitro* and *in vivo* data relevant to our current understanding of the role of DNA damage in cellular senescence and organismal ageing.

DNA DAMAGE AND CELLULAR SENESCENCE

Replicative senescence was first described in human fibroblasts as a state of permanent cell cycle arrest resulting from serial passage in culture due to a limited proliferative lifespan (5). Senescent cells undergo distinctive changes in morphology to become enlarged, flattened and granular but remain viable and metabolically active for long periods of time in culture (6,7). In addition to human fibroblasts, replicative senescence has been observed in a variety of cell types derived from many

*To whom correspondence should be addressed. Tel: +44 (0)1223 336784; Fax: +44 (0)1223 330598; Email: jhc36@cam.ac.uk
†Deceased.

species (8,9). Senescent cells can be distinguished by the presence of a biomarker—senescence associated beta-galactosidase (SA-β-gal), which is detectable at pH 6 (10,11).

DNA damage response in telomere-dependent replicative senescent cells

Most somatic cells have a finite number for population doubling and eventually become senescent because of telomere shortening due to the end replication problem (12,13). Telomeres are special chromatin structures composed of tandem repeats of the TTAGGG sequence and telomere DNA-binding factors that protect chromosomal ends from being recognized as a broken DNA end (14,15). With low or absent telomerase activity, as in the case of human diploid fibroblasts, telomeres become shorter and shorter following each round of cell division/DNA replication. Once telomeres reach a critically short length their protective structures collapse and chromosomal ends become uncapped thus triggering senescence (14,16).

The prediction that critically shortened telomeres can be recognized as a site of DNA damage was unequivocally proven by the findings that molecular markers indistinguishable from those induced by DNA damage are indeed detected in senescent human fibroblasts (17,18). These markers include nuclear foci of phosphorylated histone H2AX and their co-localization with DNA repair and DNA damage checkpoint factors such as 53BP1, MDC1 and NBS1 as well as the concomitant activation of the DNA damage inducible kinases CHK1 and CHK2 (17,18). More importantly, analysis of immunoprecipitated DNA using genomic DNA chips demonstrated that the chromosome ends of senescent cells directly contribute to the DNA damage response, and that uncapped telomeres directly associated with many DNA damage response proteins (17). Further analysis demonstrated that it is the subset of very short telomeres—devoid of most of their telomeric repeat sequences—which triggers DNA damage foci formation and terminal cell cycle arrest (19,20). Thus the functional links between telomere attrition and DNA damage response were firmly established in replicative senescence (21,22). Further evidence supporting the mediation of DNA damage response between telomere attrition and senescence were obtained by the observation that shorter telomeres and telomeric γH2AX foci were preferentially detected in early senescent cells sorted from young proliferating fibroblast cultures (23).

It is worth noting that mouse embryonic fibroblasts (MEFs), which are also widely used in the study of replicative senescence, become senescent after many fewer population doublings than human fibroblasts when cultured under standard conditions which include atmospheric (20%) oxygen. It is clear now that senescence of MEFs under this culture conditions is not due to telomere attrition but is due to their sensitivity to oxidative stress and the consequential high levels of oxidative DNA damage in 20% oxygen. Indeed it has been demonstrated that MEFs did not senesce in physiological (3%) oxygen levels (24).

DNA damage response in telomere-independent premature senescent cells

In addition to replicative senescence, a senescent phenotype can be induced prematurely in early passage cells by agents that cause DNA damage (25–29) or disrupt heterochromatin (30), by disruption of functional telomere structures (31), or by overexpression of oncogenes (32–36). These forms of premature senescence are typically induced within a period as short as several days and are not normally accompanied by telomere shortening (16,37). Despite the differences in the stressors and the lack of significant telomere shortening, there appears to be a common pathway that triggers premature senescence, which is a DNA damage response.

Sub-lethal oxidative stress such as hydrogen peroxide (H_2O_2) treatment can cause massive acute DNA double-strand breaks (DSBs) which are followed by upregulation of p53 and p21, and cell cycle arrest in the stressed cells (29,38). Much of this DNA damage can be repaired and thus the cell can re-enter the cell cycle, however some of the DNA damage persists which will eventually trigger premature senescence. Such persistent DNA damage can be increased substantially by a second H_2O_2 treatment, thus resulting in a high induction of premature senescence (29,39). In addition, oxidative stress encountered during the S-phase of the cell cycle tends to result in more DNA DSBs, higher fractions of persistent DNA damage and higher induction of premature senescence (38).

Disruption of functional protective telomere integrity by overexpression of a dominant negative TRF2 mutant results in telomere uncapping and induction of premature senescence. TRF2 is a telomere-binding protein that is essential in maintaining functional telomere structures (14). Dysfunctional, uncapped telomeres in mammalian cells caused by ectopic expression of mutant TRF2 induced a DNA damage response with DNA damage response factors, including 53BP1, γH2AX, Rad17, ATM and Mre11 being specifically associated at the dysfunctional telomeres (31). In this case, telomere lengths were not affected by the expression of mutant TRF2 suggesting that the dysfunctional state of telomeres rather than telomere shortening *per se* is an important factor in inducing a DNA damage response and premature senescence.

Recent findings show that cells that senesced in response to oncogene expression accumulated DNA damage foci. These studies demonstrated that oncogene expression caused hyperproliferation and DNA hyper-replication. Consequently, replicons refire or terminate prematurely, generating DNA breaks that initiate a DNA damage response (40–42). Oxidative stress may also contribute to DNA damage in cells overexpressing oncogenes as high levels of ROS were detected in these cells (43,44). The causative function of the DNA damage checkpoint response in induction of senescence was established by the observation that depletion of checkpoint kinases such as ATM or CHK2 resulted in by-pass of

senescence (40,41). Together, these findings established oncogene-induced DNA damage signalling as a critical mediator of oncogene-induced senescence (45).

Premature senescence can also be induced by exposure of mammalian cells to oligonucleotides homologous to the telomere 3′-overhang tandem sequence TTAGGG (T-oligos), which can be readily taken up by cells into the nucleus (46,47). The induction requires functional p53 and/or pRb pathways as the inactivation of both the p53 and pRb pathways is necessary for normal human fibroblasts to escape T-oligo-induced senescence (48). Furthermore, T-oligos can induce massive phosphorylation of H2AX (49), again suggesting that the DNA damage signalling pathway is activated in the process of T-oligo-induced premature senescence.

DNA damage response is a common mediator of cellular senescence

The compelling evidence discussed above thus suggests that cellular senescence, whether replicative senescence or premature senescence that is induced by different stressors, share a common underlying aetiology, that is, DNA damage (Figure 1). ROS are the major agents responsible for endogenous oxidative DNA damage in the cells. Therefore, any disturbance of biological systems that increase intracellular ROS levels would be expected to induce untimely senescence. Indeed, inhibition of SOD1, the copper–zinc-containing superoxide dismutase that is a major defence against ROS by detoxifying the superoxide anion, induces premature senescence in human fibroblasts (50). Overexpression of Akt, an important cell signalling molecule, was found to lead to an inhibition of the FOXO3a transcription factor and an elevation of intracellular ROS that later induced a senescence-like cell growth arrest in a p53-dependent manner (51). Increased p53 activation can trigger a senescence response with concomitant increased ROS production (52). Conversely, increasing the level of SOD delays senescence of primary fibroblasts as well as decreasing the rate of telomere shortening (53). It is beyond the scope of this review to discuss in detail the signals and pathways that activate cellular senescence following a DNA damage response, but the emerging consensus is that the signalling pathways activated by DNA damage response converge on the p53 and Rb proteins with the p53–p21–Rb pathway-mediating senescence due primarily to telomere shortening and the p16–Rb pathway is thought to mediate premature senescence [Figure 1, for reviews see (9,54–57)].

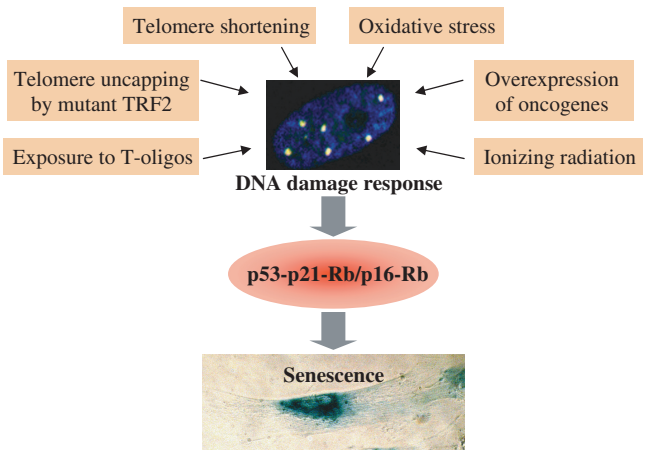

Figure 1. DNA damage response is a central mediator in triggering cellular senescence. Telomere shortening resulting from end-replication problem or stochastic loss, and various other stressors such as acute oxidative stress treatment, ionizing radiation, overexpression of oncogenes, forced telomere uncapping and exposure to T-oligos all trigger a DNA damage response during induction of cellular senescence. The signalling pathways activated by DNA damage response converge on the p53 and Rb proteins with the p53–p21–Rb pathway mediating senescence due primarily to telomere shortening while p16–Rb pathway mediates premature senescence. Images shown are DNA damage foci detected by immunofluorescence microscopy using anti-γH2AX and 53BP1 antibodies (top), and a senescent human fibroblast cell detected with SA-β-Gal (bottom).

Cellular senescence and ageing

That cellular senescence may be intimately linked with organismal ageing was supported by the following observations. First, there was a positive correlation between the replicative potential of cells in culture and the maximum lifespan of the species from which they are derived (58,59). Second, cells derived from progeroid (premature ageing) patients exhibited accelerated cellular senescence *in vitro* (60–63). Third, senescent cells are detected *in vivo* and accumulate with age. For example, senescent cells were detected and increased with age in primate skin (64,65), human vascular tissue (66–68) and rodent and human kidneys (69–71).

In addition to the above correlative observations, a recent study provided evidence that cellular senescence may play a causative role in the ageing process. Keyes *et al.* (72) found that p63 heterozygous mutant mice had a shortened lifespan and developed features of accelerated ageing. Both germline and somatically induced p63 deficiency activated widespread cellular senescence. Using an inducible tissue-specific p63 conditional model, they further showed that p63 deficiency induced cellular senescence and caused an accelerated ageing phenotype in the adult (72). This study thus established a causative link between cellular senescence and premature ageing *in vivo* (72,73).

The accumulation of senescent cells in animal organs may contribute to the ageing process by depleting the renewal capacity of tissues [path A in Figure 2, (74)] and/or by altering tissue structure and function through secretion of matrix metalloproteinases, epithelial growth factors and inflammatory cytokines which could interfere with the tissue microenvironment [path B in Figure 2, (54)]. Consequently, tissue homeostasis will be compromised which ultimately will lead to ageing (Figure 2).

DNA DAMAGE ACCUMULATES WITH AGE

According to the free radical theory of ageing, ROS play an important role in the ageing process by causing oxidative damage to biomolecules in cells (75). Here we focus our discussion on DNA damage in the ageing process. The reader is referred to other reviews for

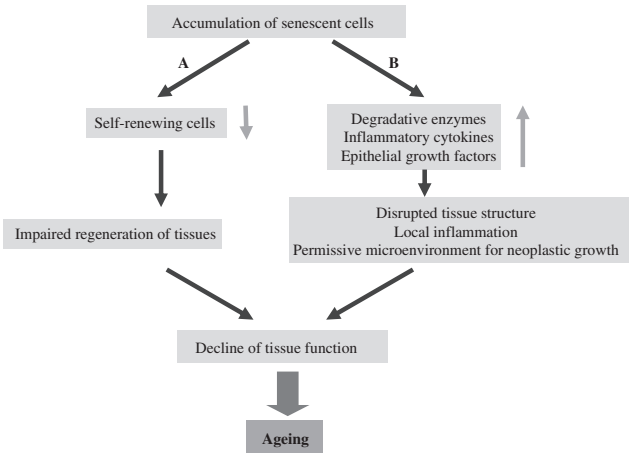

Figure 2. Two possible pathways through which cellular senescence may contribute to the ageing process. **(A)** Cellular senescence may reduce self-renewing cells, thus causing impaired regeneration of tissues. **(B)** Cellular senescence may cause disrupted tissue structure, local inflammation and permissive microenvironment for neoplastic growth through secretion of degradative enzymes, inflammatory cytokines and epithelial growth factors. Both pathways can cause compromised tissue homeostasis and function which ultimately lead to ageing.

discussion of the general role of oxidative damage to biomolecules including lipid, protein and DNA in ageing (3,4,76,77).

ROS can cause either single-strand base oxidative modification, single-strand nicks or DSBs (78). A subset of single-strand nicks may be converted to DSBs if they persist to be present during DNA replication. Among the single-strand base damage identified so far, the guanine-derived modification, 8-oxo-2-deoxyguanosine (8-oxo-dG) is the major oxidative lesion (79,80). The level of 8-oxo-dG in DNA has, therefore, been consistently used as a measure of oxidative damage to DNA in ageing studies. Although accurate measurement of 8-oxo-dG in DNA is hampered by oxidation of guanine during preparation of DNA for analysis which can result in differences in published estimates of the concentration that vary over a range of three orders of magnitude (81,82), generally, 8-oxo-dG levels increase with age in various organisms studied (4). In addition, results from a transgenic mouse model carrying a *LacZ* reporter gene showed that both *in vivo* and *in vitro* ageing were associated with an increased mutation frequency that is likely a consequence of oxidative stress (83). Lu *et al.* (84) observed that DNA damage is markedly increased in the promoters of genes with reduced expression in the aged human frontal cortex, which may cause reduced expression of genes involved in learning, memory and neuronal survival. Thus increased oxidative DNA damage was suggested to play a causative role in human brain ageing (84). By detecting γ-H2AX foci, Sedelnikova *et al.* (85) showed that persistent DNA DSBs accumulated in ageing mice and this accumulation occurred in germ as well as somatic cells. It was suggested that it is the irreparable DNA damage that may have a causal role in ageing (85), reminiscent of the causal role of irreparable DNA damage in induction of cellular senescence.

THE UNDERLYING CAUSES OF DNA DAMAGE ACCUMULATION WITH AGE

Whether DNA damage occurs and accumulates is largely determined by the levels of ROS produced and how efficiently the antioxidant defence systems remove ROS and DNA repair mechanisms repair damaged DNA. The increase of DNA damage with age may therefore be due to an imbalance between ROS generation and clearance, and decline of DNA repair mechanisms.

ROS production and antioxidant defence systems

By measuring ROS, oxidation levels of macromolecules, and a prooxidative shift in cellular redox status many studies suggest that ROS production increases with age (4). The age-associated increase in ROS production is likely due to decline in the function of electron transport chain with age. Zahn *et al.* (86) analysed changes in transcriptional profile in humans, mice and flies during ageing and found that expression of components of the electron transport chain decreased with age in all three organisms. Thus they suggested that decreased expression of the electron transport chain pathway with age might be a common marker of physiological ageing across species (86).

Age-related increase in ROS generation/oxidative stress may also be a consequence of a decline of antioxidant defence systems. However, the pattern of age-related changes in antioxidants in many tissues and species has been inconsistent. On one hand, some studies supported the notion that a decline in antioxidant defence systems occurs with ageing (87), but substantial data also exist indicating that there is no generalized decrease in antioxidant defence enzymes (88–91) with some studies even showing an age-associated increase in antioxidant enzyme activities (e.g. 92,93). Thus the correlation between antioxidant enzymes and ageing is, at best, weak and sometimes contradictory, suggesting that antioxidant enzymes may not necessarily be a limiting factor governing the degree of cellular oxidative damage with ageing.

DNA repair

DNA repair systems include base excision repair (BER) and nucleotide excision repair (NER) for single strand lesions and homologous recombination (HR) and non-homologous end-joining (NHEJ) pathways for DSBs (78,94). One important factor that causes age-associated accumulation of DNA damage may be the functional decline of DNA repair systems with age. Indeed, such declines have been observed in *in vitro* (95) and *in vivo* systems (94). For example, NHEJ becomes less efficient and more error-prone during cellular senescence (96). Decline of NHEJ efficiency has also been reported in the rat brain during ageing (97). Therefore, it was suggested that diminished efficiency and fidelity of DSB repair are responsible for age-related genomic instability (98,99). Several studies demonstrated that efficiency of NER also decreases with age as the rate of removal of UV-induced DNA lesions is slower in aged humans relative to younger

adults (100–102). This age-associated decline was shown to result from, at least in part, decreased levels of proteins that participate in the repair process (101). More recently, an age-associated decline of DNA repair efficiency including BER and NHEJ was reported in ageing rat neurons (103). In this case, age-associated compromise in BER is attributed to the deficiency of DNA polymerase β and DNA ligase in ageing neurons whereas the limiting factor(s) for compromised NHEJ remained to be identified (103). Decline of DNA repair capacity at the whole organismal level was reported recently in ageing *Caenorhabditis elegans* in which a 30–50% decrease in DNA repair in ageing adults was observed (104). Taken together, these observations suggest that age-associated accumulation of DNA damage is, at least in part, due to an age-associated decline of DNA repair capacity.

The importance of DNA repair systems in determining longevity has been demonstrated convincingly in premature ageing patients (e.g. Werner and Cockayne syndromes). Werner syndrome (WS) is a segmental progeroid disease characterized by acceleration of specific age-related phenotypes and increased cancer due to loss of a helicase protein known as WRN. It was suggested that increased accumulation of DNA strand breaks as well as dysfunctional telomeres and resulting premature senescence play a causative role in the WS (63,105). This notion is supported by the recent findings that WS cells tend to have an increased accumulation of DSBs and enhanced genomic instability including telomere dysfunction (106) and that replication-associated telomere loss was responsible for chromosomal aberrations in WS fibroblasts (107). The biological significance of functional DNA damage repair is further underpinned by the fact that many other progeroid syndromes including Cockayne syndrome and trichothiodystrophy, are NER-related disorders (108,109). The connections between impaired BER and human disease are fewer than NER-related disorders. This is likely due to either the embryonic lethality of defects in essential BER components or the multitude of back-up systems (i.e. redundancy of BER enzymes) in the removal of oxidatively damaged bases from DNA, which may reflect the critical nature of BER in maintaining genome integrity (110–112).

An emerging third link—age-related shifts in DSB repair pathway usage

In addition to the observations that DNA damage increases and DNA repair capacity decreases with age and that defects in DNA repair genes are associated with premature ageing, now a third link has emerged: ageing is also associated with changes in DSB repair pathway usage, from simpler NHEJ in younger organisms to HR in the aged ones. Using a transgenic repair reporter construct Preston *et al.* (113,114) discovered that in sperm from male *Drosophila* the predominant mechanism by which DNA DSBs are repaired changes dramatically as the male ages. Interestingly they found that the age-related changes in DNA repair were not an overall increase or decrease in repair capacity, but rather a shift in the relative usage of repair mechanisms such that younger organisms repair DSBs primarily by NHEJ or single-strand annealing (SSA) whereas in older individuals HR becomes the predominant repair process (115). Given the fact that DNA damage accumulates with age, it is surprising that ageing is correlated with reduced usage of NHEJ and SSA, which are actually error-prone and increased usage of HR which is the more accurate repair pathway. The authors suggest that their findings may be in keeping with the 'antagonistic pleiotropy' hypothesis (116) in that the use of simpler end joining processes to repair breaks avoids time-consuming DNA synthesis, thus allowing more rapid development and offering a significant competitive advantage for most species. However, the use of these error-prone pathways at early ages may result in faster accumulation of DNA damage with deleterious consequences later in life (115).

The mechanism by which relative usage of DSBs repair shifts with age is currently unknown although it is speculated that the shift might be due to age-related changes in expression levels and/or activities of components of the repair pathways (117). Also, the generality of age-related changes in DSB repair pathway usage remains to be established.

CAUSAL OR CORRELATIVE? EVIDENCE FROM EXPERIMENTAL INTERVENTIONS

Although numerous reports indicate that DNA damage increases with age, the question of whether DNA damage is a causative agent of ageing or it is merely a correlative accumulation with ageing cannot be answered by descriptive observations. In order to address this question, experimental interventions that can alter lifespan are needed. These experimental interventions include genetic and non-genetic approaches.

Evidence from non-genetic approaches

That DNA damage may play a causal role in the ageing process was supported by observations in CR (Caloric restriction) animals. Various studies have reported reductions in steady-state oxidative damage to cellular macromolecules including DNA in CR animals (118,119). The reductions in oxidative damage by CR has been attributed to a decline in the rate of ROS generation and/or enhanced repair mechanism (118,119). These observations thus provide a link between attenuated DNA damage/enhanced DNA repair and lifespan extension by CR.

Indirect evidence supporting the role of oxidative DNA damage in ageing is also available from pharmacological intervention studies. Synthetic antioxidant enzyme mimetics such as EUK-8, EUK-134 and EUK-189, which have broad-spectrum efficacy against both superoxide and hydrogen peroxide showed lifespan extension effects in *C. elegans* (120). Moreover, treatment of SOD2 (the mitochondrial form of SOD) nullizygous mice with these mimetics attenuated mitochondrial defects and extended their lifespan by 3-fold (121). Similarly, administration of antioxidant mimetics to ATM-deficient mice suppressed oxidative DNA damage and DNA deletions, and increased longevity (122).

Recently, caloric restriction mimetics such as resveratrol and related polyphenol compounds have been shown to extend lifespan of yeast, *C. elegans* and *Drosophila* (123,124). Moreover, resveratrol was also found to exert its beneficial biological functions and promote survival in mice (125–127). Evidence from *in vitro* and *in vivo* studies demonstrated that resveratrol can effectively scavenge ROS, upregulate the expression of antioxidant enzymes and increase resistance to oxidative stress (128–130). These findings support the notion that oxidative damage is a major determinant of lifespan and that it can be counteracted by pharmacological interventions.

Evidence from genetic approaches

Genetic models that show lifespan extension usually involve overexpression of antioxidant enzymes or altering gene expression in a manner that increases resistance to oxidative stress. For example, overexpression of SOD1 and SOD2 in *Drosophila* (131–134) and yeast (135,136) extended lifespan. Positive effects of overexpression of antioxidant enzymes on longevity have also been observed in mammalian models. Schriner *et al.* (137) showed that transgenic mice overexpressing catalase in mitochondria increased both median and maximum lifespan, which was accompanied by decreased H_2O_2 production and reduced oxidative damage. The overexpression of other antioxidant enzymes including glutamate-cysteine ligase (138), methionine sulphoxide reductase (139) and thioredoxin (140) also can extend lifespan. Whether extension of lifespan in the model organisms overexpressing antioxidant enzymes is through mitigating cellular senescence remains to be established.

In addition to the genetic models overexpressing antioxidant enzymes, various other genetic models with extended lifespan have been reported in recent years. These genetic models include disrupted expression of GH and insulin/IGF1 signalling pathways (141–146), $p66^{sch}$ (147,148) and clk-1/mclk-1 (149,150), and overexpression of Klotho (151,152), MST/CST (153) and FOXOs (154,155) as well as enhanced JNK signalling (156,157). A common theme that is repeatedly identified in these genetic models is the enhanced expression of antioxidant defence systems, increased resistance to oxidative stress and reduced oxidative damage.

Alongside the genetic models of extended lifespan, there are genetic models of premature ageing. These models typically involve disrupted expression of proteins that play a role in the maintenance of DNA (including telomere) integrity such as WRN, Ku80, ATM and ERCC1 (158–160). Evidence supporting the causal role of genomic instability in ageing has been further obtained from two recent mutant mouse models. Mostoslavsky *et al.* (161) showed that deficiency of Sirt6, one of seven mammalian Sir2 family members caused defective BER, elevated levels of spontaneous genomic instability and led to premature ageing. Wang *et al.* (162) reported that Cdc42 GTPase-activating protein deficiency can cause reduced DNA damage repair ability, increased genomic abnormalities, premature senescence and ultimately premature ageing phenotypes in mice. This study thus provided an interesting link between genomic instability, cellular senescence and ageing (162).

Two recent reports using progeroid mouse models provided a very important link between DNA damage and ageing. Niedernhofer *et al.* (163) showed that $Ercc1^{-/-}$ mice recapitulated the progeroid syndrome of a human patient. Comprehensive analysis of gene expression in the $Ercc1^{-/-}$ mice liver revealed a broad spectrum of changes as compared with littermate controls. These changes included a general decrease in the activity of hormonal pathways involved in the regulation of metabolism, such as GH/IGF1 signalling, and increased activity in antioxidant and DNA repair pathways (163). By using a different NER-deficient mouse model—$Csb^{m/m}/Xpa^{-/-}$ double-mutant mice, van der Pluijm *et al.* (164) found similar gene expression changes as seen in $Ercc1^{-/-}$ mice, including suppressed GH/IGF1 endocrine signalling and the upregulation of antioxidant defence genes. These two studies also showed that the suppression of GH/IGF1 signalling could also be induced by chronic exposure to DNA damage agents. Strikingly the gene expression pattern observed in NER-deficient and in mutagen-treated mice in these two studies is reminiscent of the array of changes previously reported in long-lived mutant *C. elegans* and CR mice (144). The authors suggest that this seemingly paradoxical observation may be explained by postulating that DNA damage, whatever the causes, triggers a common, highly conserved stress response which is systemic suppression of the GH/IGF1 hormone axis. This in turn leads to metabolic changes that shift energy usage from growth and proliferation to protective maintenance, minimizing further damage. Progeroid mice resulting from DNA-repair deficiency thus mount the same protective response, but cannot fully counter the consequences of a high load of DNA damage. Consequently excess DNA damage and correspondingly high levels of mutagenesis, cellular senescence and cell death may conspire to promote progeroid changes and disease pathogenesis (165).

Recent studies also provided evidence that links DNA damage, declined stem cell functionality and ageing. Mice deficient in genomic maintenance pathways such as NER, NHEJ and telomere maintenance showed decreased stem cell functional capacity including loss of reconstitution and proliferative potential, diminished self-renewal, increased apoptosis and ultimate functional exhaustion (166). Similarly mice with diminished DNA DSB repair caused a progressive loss of haematopoietic stem cell and bone marrow cellularity during ageing, and severely impaired stem cell function in tissue culture and transplantation (167). Furthermore, deficiency in DNA damage response by deletion of ATR in adult mice caused rapid premature ageing, resulting from reductions in tissue-specific stem and progenitor cells, and exhaustion of tissue renewal and homeostatic capacity due to forced regeneration pressure imposed in residual ATR-competent cells (168,169).

Conflicting data

Despite the large body of evidence from the experimental intervention studies supporting the critically important

role of DNA damage in the ageing process, there are reports that either cast doubts on the effectiveness of some antioxidant mimetics or show data contradictory to the correlative relationship between antioxidant activities, DNA damage and ageing. For example, no effects of EUK-8 and EUK-134 could be found in house flies that were treated with various concentrations of the mimetics (170). Administration of these mimetics to *C. elegans* increased cellular SOD activity in a dose-dependent manner, but failed to extend lifespan (171). More recently, Partridge and co-workers tested the effects of EUK-8 and -134 and MitoQ, one of a new class of mitochondria-targeted antioxidants (172) in *Drosophila* under various conditions (173). They found that although the three drugs did significantly increase the lifespan of SOD-deficient flies and improved their tolerance to paraquat stress these antioxidant drugs all failed to increase the lifespan or to rescue the paraquat sensitivity of wild-type flies (173). Moreover, in an earlier study they found that although CR extended lifespan in *Drosophila*, there was no significant difference in mitochondrial ROS production compared with controls and that overexpression of mitochondrial adenine nucleotide translocase lowered membrane potential and ROS production but did not extend lifespan (174). Furthermore overexpression of SOD in *Drosophila* failed to extend lifespan in some studies, and in some cases even shortened lifespan (175,176). Conversely, heterozygous MnSOD knockout mice showed decreased MnSOD activity (177), increased sensitivity to oxidative stress (178), increased oxidative DNA damage and even a higher incidence of cancer, but the lifespan was not affected (179).

MITOCHONDRIAL DNA (mtDNA) DAMAGE AND AGEING

Mitochondria contain their own genome, which is a circular double-stranded DNA molecule of ~16 kb (mtDNA). Mammalian mtDNA contains 37 genes, which code for 13 polypeptide components of the respiratory chain as well as rRNAs and tRNAs necessary for intramitochondrial protein synthesis (180). As mitochondria are the major source of ROS, together with the fact that mitochondria do not have the enzymes necessary for NER and protective histone wrapping, it has long been suspected that mtDNA is the prime and vulnerable target of ROS attack (181). It is also suspected that mtDNA damage, if not repaired, leads to disruption of the electron transport chain and production of more ROS, which, in turn, leads to further mtDNA damage, hence the concept of a 'vicious cycle' of ROS production and mtDNA damage. The importance of mtDNA damage in ageing and age-associated diseases have been supported by the observations that mtDNA damage (including point mutations and deletions) increases with age and mitochondrial dysfunction is a common aetiology of many age-associated neurodegenerative diseases (180–183).

To address whether defective mtDNA plays a causative role in the ageing process, Trifunovic *et al.* (184) created a mutant mouse model in which the proof-reading ability of mtDNA polymerase is lost by replacing the critical aspartate residue with alanine in one of the three exonuclease domains. They found that the mutant mice showed elevated levels of point mutations as well as increased amounts of deletions and that this increase in somatic mtDNA was associated with reduced lifespan and early onset of ageing-related phenotypes (184). Apparently, this study provided a causative link between mtDNA mutations and premature ageing phenotypes in mammals. In order to see whether the premature ageing phenotypes in the mutant mice were due to increased oxidative stress that might be caused by increased mtDNA mutations Kujoth *et al.* (185) investigated markers of oxidative stress, including levels of protein carbonyl, F2-isoprostanes and 8-oxo-dG (oxidative damage to DNA) and 8-oxo-G (oxidative damage to RNA), in a similar mutant mouse model. Surprisingly, they found that accumulation of mtDNA mutations was not associated with increased mitochondrial H_2O_2 production or increased markers of oxidative stress, but was correlated with the induction of apoptotic markers. The levels of apoptotic markers were also found to increase during ageing in normal mice. Therefore, they suggested that apoptosis and subsequent loss of irreplaceable cells might be an important mechanism of ageing in mammals (183,186). Trifunovic *et al.* (187) found in a subsequent study that increased mtDNA mutations indeed did not affect ROS production in their mutant mice. Thus the premature ageing phenotypes in mtDNA mutant mice were not caused by a vicious cycle of massively increased oxidative stress as initially suspected. These findings also provided strong evidence that argue against any direct role of oxidative stress in the premature ageing process in the mutant mouse models (188,189).

Does premature ageing of the mtDNA mutant mouse prove that mtDNA mutations are involved in the natural ageing process? This question was raised by Khrapko *et al.* (190) based on the fact that the levels of mutations in the mutant mice are typically more than an order of magnitude higher than typical levels in aged humans. By using a more accurate assay, Vermulst *et al.* (191) found that mtDNA mutations increased with age in both wild-type and mutant mice with the mutation frequency in homozygous mutant mice being 2500-fold higher than in wild-type mice. Remarkably, heterozygous mice showed an ~500-fold higher mutation burden than age-matched normal mice, with no obvious features of premature ageing (191). This study indicated that mitochondrial mutations do not limit the lifespan of wild-type mice, thus casting doubt on a causal role in normal ageing (192).

CONCLUSIONS

An accumulated large body of evidence has demonstrated beyond doubt that DNA damage is a crucial mediator for various stresses during cellular senescence regardless of whether they are telomere dependent or independent and that oxidative DNA damage accumulates with age. The age-associated accumulation of DNA damage is attributable to an age-related increase in ROS production and a

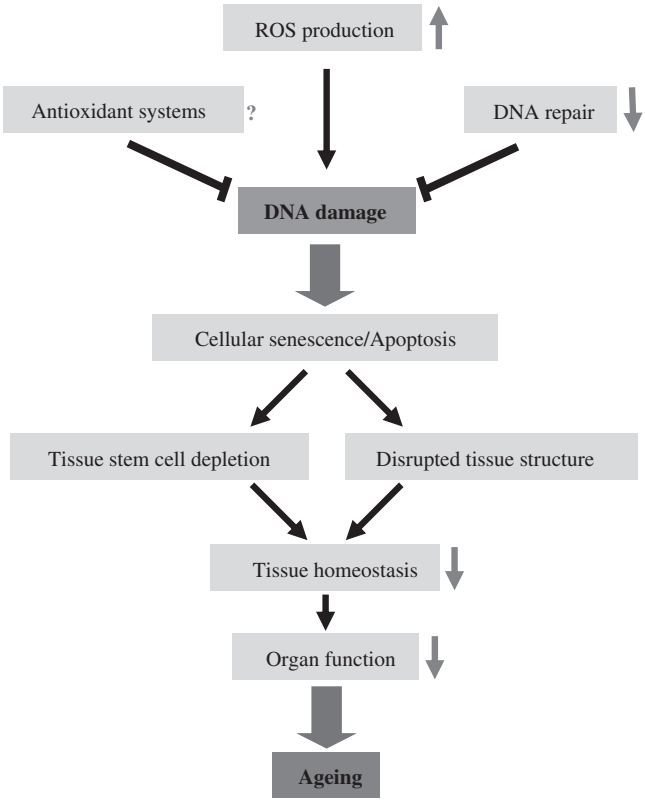

Figure 3. Major components that contribute to age-related accumulation of DNA damage and the subsequent consequences that lead to ageing. Age-related increase in ROS production and decline in DNA repair capacity have been identified as two major factors that cause age-associated accumulation of DNA damage. It is less clear as to how the antioxidant defence systems influence increased accumulation of DNA damage during ageing. At the cellular levels DNA damage results in cellular senescence or apoptosis, which in turn lead to compromised tissue homeostasis through stem cell depletion and/or disrupted tissue structure as detailed in Figure 2. Ultimately organ function declines and phenotypical features of ageing manifest at organismal level.

decline in DNA repair capacity although how changes in antioxidant defence systems contribute remains less clear. Increasingly, experimental interventions, particularly genetic animal models serve to provide valuable insight into underlying mechanisms at both molecular and cellular levels. Thus it is now clear that at the cellular level DNA damage results in cellular senescence or apoptosis, which in turn leads to compromised tissue homeostasis, most likely through diminished self-renewal or altered tissue structure. Ultimately phenotypical features of ageing manifest at organismal level (Figure 3).

With the accelerated pace of genetic models being created and more sophisticated approaches (e.g. developmental stage-specific, tissue-specific and cell type-specific as well as dose controllable) being used, it is hoped that new data will continue to provide new links between the components that are implicated in the ageing process (e.g. DNA damage, insulin/IGF1 signalling and metabolism), to strengthen the weak links (e.g. cellular senescence and ageing) and to enrich the established links (e.g. DNA repair capacity and ageing). At present, the correlative relationship between DNA damage and ageing is strong and a causative role of compromised DNA maintenance or accelerated mtDNA mutations in premature ageing is convincing. However, whether DNA damage plays a causative role in normal ageing still remains to be established. It is hoped that this question may be addressed by creating an animal model with enhanced DNA repair capacity or enhanced DNA polymerase proofreading capacity.

Hayflick (193), who first described cellular senescence over four decades ago, recently keenly declared that 'Biological ageing is no longer an unsolved problem'. It is hoped that with the science of ageing rapidly growing in depth, breadth and molecular detail, one day it will be possible to declare 'Mechanisms of biological ageing are no longer an unsolved problem'.

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